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**APPLICATION
FOR
UNITED STATES LETTERS PATENT**

TITLE: PLANTS HAVING MUTANT SEQUENCES THAT CONFER
ALTERED FATTY ACID PROFILES

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PLANTS HAVING MUTANT SEQUENCES THAT CONFER ALTERED
FATTY ACID PROFILES

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Cross Reference To Related Applications

This application is a continuation-in-part of U.S. Serial No. 08/416,497, filed April 4, 1995, which is a continuation of U.S. Serial No. 08/170,886, filed December 21, 1993, now abandoned, which is a continuation-in-part of U.S. Serial No. 07/739,965, filed August 5, 1991, which is a continuation-in-part of U.S. Serial No. 07/575,542, filed August 30, 1990.

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Technical Field

This invention relates to *Brassica* seeds and plants having mutant sequences which confer altered fatty acid profiles on the seed oil. More particularly, the invention relates to mutant delta-12 and delta-15 fatty acid desaturase sequences in such plants which confer such profiles.

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Background of the Invention

Diets high in saturated fats increase low density lipoproteins (LDL) which mediate the deposition of cholesterol on blood vessels. High plasma levels of serum cholesterol are closely correlated with atherosclerosis and coronary heart disease (Conner et al., Coronary Heart Disease: Prevention, Complications, and Treatment, pp. 43-64, 1985). By producing oilseed *Brassica* varieties with reduced levels of individual and total saturated fats in the seed oil, oil-based food products which contain less saturated fats can be produced. Such products will benefit public health by reducing the incidence of atherosclerosis and coronary heart disease.

The dietary effects of monounsaturated fats have also been shown to have dramatic effects on health. Oleic acid, the only monounsaturated fat in most edible vegetable oils, lowers LDL as effectively as linoleic acid, but does 5 not affect high density lipoproteins (HDL) levels (Mattson, F.H., J. Am. Diet. Assoc., 89:387-391, 1989; Mensink et al., New England J. Med., 321:436-441, 1989). Oleic acid is at least as effective in lowering plasma cholesterol as a diet low in fat and high in carbohydrates (Grundy, S.M., New 10 England J. Med., 314:745-748, 1986; Mensink et al., New England J. Med., 321:436-441, 1989). In fact, a high oleic acid diet is preferable to low fat, high carbohydrate diets for diabetics (Garg et al., New England J. Med., 319:829-834, 1988). Diets high in monounsaturated fats are also 15 correlated with reduced systolic blood pressure (Williams et al., J. Am. Med. Assoc., 257:3251-3256, 1987). Epidemiological studies have demonstrated that the "Mediterranean" diet, which is high in fat and monounsaturates, is not associated with coronary heart 20 disease (Keys, A., Circulation, 44(Suppl):1, 1970).

Many breeding studies have been conducted to improve the fatty acid profile of *Brassica* varieties. Pleines and Freidt, Fat Sci. Technol., 90(5), 167-171 (1988) describe plant lines with reduced C_{18:3} levels (2.5-5.8%) combined 25 with high oleic content (73-79%). Rakow and McGregor, J. Amer. Oil Chem. Soc., 50, 400-403 (Oct. 1973) discuss problems associated with selecting mutants for linoleic and linolenic acids. In. Can. J. Plant Sci., 68, 509-511 (Apr. 1988) Stellar summer rape producing seed oil with 3% 30 linolenic acid and 28% linoleic acid is disclosed. Roy and Tarr, Z. Pflanzenzuchtg, 95(3), 201-209 (1985) teaches transfer of genes through an interspecific cross from *Brassica juncea* into *Brassica napus* resulting in a

reconstituted line combining high linoleic with low linolenic acid content. Roy and Tarr, Plant Breeding, 98, 89-96 (1987) discuss prospects for development of *B. napus* L. having improved linolenic and linolenic acid content.

5 European Patent application 323,751 published July 12, 1989 discloses seeds and oils having greater than 79% oleic acid combined with less than 3.5% linolenic acid. Canvin, Can. J. Botany, 43, 63-69 (1965) discusses the effect of temperature on the fatty acid composition of oils from

10 several seed crops including rapeseed.

Mutations typically are induced with extremely high doses of radiation and/or chemical mutagens (Gaul, H. Radiation Botany (1964) 4:155-232). High dose levels which exceed LD50, and typically reach LD90, led to maximum

15 achievable mutation rates. In mutation breeding of *Brassica* varieties high levels of chemical mutagens alone or combined with radiation have induced a limited number of fatty acid mutations (Rakow, G.Z. Pflanzenzuchtg (1973) 69:62-82). The low α -linolenic acid mutation derived from the Rakow

20 mutation breeding program did not have direct commercial application because of low seed yield. The first commercial cultivar using the low α -linolenic acid mutation derived in 1973 was released in 1988 as the variety Stellar (Scarth, R. et al., Can. J. Plant Sci. (1988) 68:509-511). Stellar was

25 20% lower yielding than commercial cultivars at the time of its release.

Canola-quality oilseed *Brassica* varieties with reduced levels of saturated fatty acids in the seed oil could be used to produce food products which promote

30 cardiovascular health. Canola lines which are individually low in palmitic and stearic acid content or low in combination will reduce the levels of saturated fatty acids. Similarly, *Brassica* varieties with increased monounsaturate

levels in the seed oil, and products derived from such oil, would improve lipid nutrition. Canola lines which are low in linoleic acid tend to have high oleic acid content, and can be used in the development of varieties having even
5 higher oleic acid content.

Increased palmitic acid content provides a functional improvement in food applications. Oils high in palmitic acid content are particularly useful in the formulation of margarines. Thus, there is a need for
10 manufacturing purposes for oils high in palmitic acid content.

Decreased α -linolenic acid content provides a functional improvement in food applications. Oils which are low in linolenic acid have increased stability. The rate of
15 oxidation of lipid fatty acids increases with higher levels of linolenic acid leading to off-flavors and off-odors in foods. There is a need in the food industry for oils low in alpha linolenic acid.

Delta-12 fatty acid desaturase (also known as oleic acid desaturase) is involved in the enzymatic conversion of oleic acid to linoleic acid. Delta-15 fatty acid desaturase (also known as linoleic acid desaturase) is involved in the enzymatic conversion of linoleic acid to α -linolenic acid.
20 A microsomal delta-12 desaturase has been cloned and characterized using T-DNA tagging. Okuley, et al., Plant Cell 6:147-158 (1994). The nucleotide sequences of higher plant genes encoding microsomal delta-12 fatty acid desaturase are described in Lightner et al., WO94/11516.
25 Sequences of higher plant genes encoding microsomal and plastid delta-15 fatty acid desaturases are disclosed in Yadav, N., et al., Plant Physiol., 103:467-476 (1993), WO 93/11245 and Arondel, V. et al., Science, 258:1353-1355 (1992). However, there are no teachings that disclose
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mutations in delta-12 or delta-15 fatty acid desaturase coding sequences from plants. Furthermore, no methods have been described for developing plant lines that contain delta-12 or delta-15 fatty acid desaturase gene sequence 5 mutations effective for altering the fatty acid composition of seeds.

Summary of the Invention

The present invention comprises canola seeds, plant lines producing seeds, and plants producing seed, said seeds 10 having a maximum content of FDA saturates of about 5% and a maximum erucic acid content of about 2% based upon total extractable oil and belonging to a line in which said saturates content has been stabilized for both the generation to which the seed belongs and its parent 15 generation. Progeny of said seeds and canola oil having a maximum erucic acid content of about 2%, based upon total extractable oil, are additional aspects of this invention. Preferred are seeds, plant lines producing seeds, and plants producing seeds, said seeds having an FDA saturates content 20 of from about 4.2% to about 5.0% based upon total extractable oil.

The present invention further comprises *Brassica* seeds, plant lines producing seeds, and plants producing seeds, said seeds having a minimum oleic acid content of 25 about 71% based upon total extractable oil and belonging to a line in which said oleic acid content has been stabilized for both the generation to which the seed belongs and its parent generation. A further aspect of this invention is such high oleic acid seeds additionally having a maximum 30 erucic acid content of about 2% based upon total extractable oil. Progeny of said seeds; and *Brassica* oil having 1) a minimum oleic acid content of about 71% or 2) a minimum

oleic acid content of about 71% and a maximum erucic content of about 2% are also included in this invention. Preferred are seeds, plant lines producing seeds, and plants producing seeds, said seeds having an oleic acid content of from about 5 71.2% to about 78.3% based upon total extractable oil.

The present invention further comprises canola seeds, plant lines producing seeds, and plants producing seeds, said seeds having a maximum linoleic acid content of about 14% and a maximum erucic acid content of about 2% based upon total extractable oil and belonging to a line in which said acid content is stabilized for both the 10 generation to which the seed belongs and its parent generation. Progeny of said seeds and canola oil having a maximum linoleic acid content of about 14% and a maximum 15 erucic acid content of about 2%, are additional aspects of this invention. Preferred are seeds, plant lines producing seeds, and plants producing seeds, said seeds having a linoleic acid content of from about 8.4% to about 9.4% based upon total extractable oil.

The present invention further comprises *Brassica* seeds, plant lines producing seeds, and plants producing seeds, said seeds having a maximum palmitic acid content of about 3.5% and a maximum erucic acid content of about 2% based on total extractable oil and belonging to a line in 20 which said acid content is stabilized for both the generation to which the seed belongs and its parent 25 generation. Progeny of said seeds and canola having a maximum palmitic acid content of about 3.5% and a maximum erucic acid content of about 2%, are additional aspects of 30 this invention. Preferred are seeds, plant lines producing seeds, and plants producing seeds, said seeds having a palmitic acid content of from about 2.7% to about 3.1% based upon total extractable oil.

The present invention further comprises *Brassica* seeds, plant lines producing seeds, and plants producing seeds, said seeds having a minimum palmitic acid content of about 9.0% based upon total extractable oil and belonging to 5 a line in which said acid content is stabilized for both the generation to which the seed belongs and its parent generation. A further aspect of this invention is such high palmitic acid seeds additionally having a maximum erucic acid content of about 2% based upon total extractable oil.

10 Progeny of said seeds; and *Brassica* oil having 1) a minimum palmitic acid content of about 9.0%, or 2) a minimum palmitic acid content of about 9.0% and a maximum erucic acid content of about 2% are also included in this invention. Preferred are seeds, plant lines producing 15 seeds, and plants producing seeds, said seeds having a palmitic acid content of from about 9.1% to about 11.7% based upon total extractable oil.

The present invention further comprises *Brassica* seeds, plant lines producing seeds, and plants producing 20 seeds, said seeds having a maximum stearic acid content of about 1.1% based upon total extractable oil and belonging to a line in which said acid content is stabilized for both the generation to which the seed belongs and its parent generation. Progeny of said seeds have a canola oil having 25 a maximum stearic acid content of about 1.1% and maximum erucic acid content of about 2%. Preferred are seeds, plant lines producing seeds, and plants producing seeds having a palmitic acid content of from about 0.8% to about 1.1% based on total extractable oil.

30 The present invention further comprises *Brassica* seeds, plant lines producing seeds, and plants producing seeds, said seeds having a sum of linoleic acid content and linolenic acid content of a maximum of about 14% based upon

total extractable oil and belonging to a line in which said acid content is stabilized for both the generation to which the seed belongs and its parent generation. Progeny of said seeds have a canola oil having a sum of linoleic acid
5 content and linolenic acid content of a maximum of about 14% and a maximum erucic acid content of about 2%. Preferred are seeds, plant lines producing seeds, and plants producing seeds having a sum of linoleic acid content and linolenic acid content of from about 11.8% to about 12.5% based on
10 total extractable oil.

The invention further comprises *Brassicaceae* or *Helianthus* seeds, plants and plant lines having at least one mutation that controls the levels of unsaturated fatty acids in plants. One embodiment of the invention is an isolated
15 nucleic acid fragment comprising a nucleotide sequence encoding a mutant delta-12 fatty acid desaturase conferring increased levels of oleic acid when the fragment is present in a plant. A preferred sequence comprises a mutant sequence as shown in SEQ ID NO:3. Another embodiment of the
20 invention is an isolated nucleic acid fragment comprising a nucleotide sequence encoding a mutant delta-15 fatty acid desaturase. A plant in this embodiment may be soybean, oilseed *Brassica* species, sunflower, castor bean or corn. The mutant sequence may be derived from, for example, a
25 *Brassica napus*, *Brassica rapa*, *Brassica juncea* or *Helianthus* delta-12 or delta-15 gene.

Another embodiment of the invention involves a method of producing a *Brassicaceae* or *Helianthus* plant line comprising the steps of: (a) inducing mutagenesis in cells
30 of a starting variety of a *Brassicaceae* or *Helianthus* species; (b) obtaining progeny plants from the mutagenized cells; (c) identifying progeny plants that contain a

mutation in a delta-12 or delta-15 fatty acid desaturase gene; and (d) producing a plant line by selfing.

Yet another embodiment of the invention involves a method of producing plant lines containing altered levels of unsaturated fatty acids comprising: (a) crossing a first plant with a second plant having a mutant delta-12 or delta-15 fatty acid desaturase; (b) obtaining seeds from the cross of step (a); (c) growing fertile plants from such seeds; (d) obtaining progeny seed the plants of step (c); and (e) identifying those seeds among the progeny that have altered fatty acid composition. Suitable plants are soybean, rapeseed, sunflower, safflower, castor bean and corn. Preferred plants are rapeseed and sunflower.

The invention is also embodied in vegetable oil obtained from plants disclosed herein, which vegetable oil has an altered fatty acid composition.

Brief Description of the Figures

Figure 1 is a histogram showing the frequency distribution of seed oil oleic acid ($C_{18:1}$) content in a segregating population of a Q508 X Westar cross. The bar labeled WSGA 1A represents the $C_{18:1}$ content of the Westar parent. The bar labeled Q508 represents the $C_{18:1}$ content of the Q508 parent.

Description of the Preferred Embodiments

The U.S. Food and Drug Administration defines saturated fatty acids as the sum of lauric ($C_{12:0}$), myristic ($C_{14:0}$), palmitic ($C_{16:0}$) and stearic ($C_{18:0}$) acids. The term "FDA saturates" as used herein means this above-defined sum. Unless total saturate content is specified, the saturated fatty acid values expressed here include only "FDA saturates."

All percent fatty acids herein are percent by weight of the oil of which the fatty acid is a component.

As used herein, a "line" is a group of plants that display little or no genetic variation between individuals 5 for at least one trait. Such lines may be created by several generations of self-pollination and selection, or vegetative propagation from a single parent using tissue or cell culture techniques. As used herein, the term "variety" refers to a line which is used for commercial production.

10 The term "mutagenesis" refers to the use of a mutagenic agent to induce random genetic mutations within a population of individuals. The treated population, or a subsequent generation of that population, is then screened for usable trait(s) that result from the mutations. A
15 "population" is any group of individuals that share a common gene pool. As used herein " M_0 " is untreated seed. As used herein, " M_1 " is the seed (and resulting plants) exposed to a mutagenic agent, while " M_2 " is the progeny (seeds and plants) of self-pollinated M_1 plants, " M_3 " is the progeny of self-
20 pollinated M_2 plants, and " M_4 " is the progeny of self-pollinated M_3 plants. " M_5 " is the progeny of self-pollinated M_4 plants. " M_6 ", " M_7 ", etc. are each the progeny of self-pollinated plants of the previous generation. The term "selfed" as used herein means self-pollinated.

25 "Stability" or "stable" as used herein means that with respect to a given fatty acid component, the component is maintained from generation to generation for at least two generations and preferably at least three generations at substantially the same level, e.g., preferably $\pm 5\%$. The
30 method of invention is capable of creating lines with improved fatty acid compositions stable up to $\pm 5\%$ from generation to generation. The above stability may be affected by temperature, location, stress and time of

planting. Thus, comparison of fatty acid profiles should be made from seeds produced under similar growing conditions. Stability may be measured based on knowledge of prior generation.

5 Intensive breeding has produced *Brassica* plants whose seed oil contains less than 2% erucic acid. The same varieties have also been bred so that the defatted meal contains less than 30 μmol glucosinolates/gram. "Canola" as used herein refers to plant variety seed or oil which 10 contains less than 2% erucic acid ($C_{22:1}$), and meal with less than 30 μmol glucosinolates/gram.

Applicants have discovered plants with mutations in a delta-12 fatty acid desaturase gene. Such plants have useful alterations in the fatty acid compositions of the 15 seed oil. Such mutations confer, for example, an elevated oleic acid content, a decreased, stabilized linoleic acid content, or both elevated oleic acid and decreased, stabilized linoleic acid content.

Applicants have further discovered plants with 20 mutations in a delta-15 fatty acid desaturase gene. Such plants have useful alterations in the fatty acid composition of the seed oil, e.g., a decreased, stabilized level of α -linolenic acid.

Applicants have further discovered isolated nucleic 25 acid fragments comprising sequences that carry mutations within the coding sequence of delta-12 or delta-15 desaturases. The mutations confer desirable alterations in fatty acid levels in the seed oil of plants carrying such mutations. Delta-12 fatty acid desaturase is also known as omega-6 fatty acid desaturase and is sometimes referred to herein as 12-DES. Delta-15 fatty acid desaturase is also known ~~as~~¹⁵ omega-3 fatty acid desaturase and is sometimes referred to herein as 15-DES.

A nucleic acid fragment of the invention contains a mutation in a microsomal delta-12 fatty acid desaturase coding sequence or in a microsomal delta-15 fatty acid desaturase coding sequence. Such a mutation renders the resulting desaturase gene product non-functional in plants, relative to the function of the gene product encoded by the wild-type sequence. The non-functionality of the 12-DES gene product can be inferred from the decreased level of reaction product (linoleic acid) and increased level of substrate (oleic acid) in plant tissues expressing the mutant sequence, compared to the corresponding levels in plant tissues expressing the wild-type sequence. The non-functionality of the 15-DES gene product can be inferred from the decreased level of reaction product (α -linolenic acid) and the increased level of substrate (linoleic acid) in plant tissues expressing the mutant sequence, compared to the corresponding levels in plant tissues expressing the wild-type sequence.

A nucleic acid fragment of the invention may comprise a portion of the coding sequence, e.g., at least 20 nucleotides, provided that the fragment contains at least one mutation in the coding sequence. In one embodiment, a nucleic acid fragment of the invention comprises the full length coding sequence of a mutant delta-12 or mutant delta-15 fatty acid desaturase.

A mutation in a nucleic acid fragment of the invention may be in any portion of the coding sequence that renders the resulting gene product non-functional. Suitable types of mutations include, without limitation, insertions of nucleotides, deletions of nucleotides, or transitions and transversions in the wild-type coding sequence. Such mutations result in insertions of one or more amino acids, deletions of one or more amino acids, and non-conservative

amino acid substitutions in the corresponding gene product. In some embodiments, the sequence of a nucleic acid fragment may comprise more than one mutation or more than one type of mutation.

5 Insertion or deletion of amino acids in a coding sequence may, for example, disrupt the conformation of essential alpha-helical or beta-pleated sheet regions of the resulting gene product. Amino acid insertions or deletions may also disrupt binding or catalytic sites important for
10 gene product activity. It is known in the art that the insertion or deletion of a larger number of contiguous amino acids is more likely to render the gene product non-functional, compared to a smaller number of inserted or deleted amino acids.

15 Non-conservative amino acid substitutions may replace an amino acid of one class with an amino acid of a different class. Non-conservative substitutions may make a substantial change in the charge or hydrophobicity of the gene product. Non-conservative amino acid substitutions may
20 also make a substantial change in the bulk of the residue side chain, e.g., substituting an alanyl residue for a isoleucyl residue.

61 Examples of non-conservative substitutions include a basic amino acid for a non-polar amino acid, or a polar
25 amino acid for an acidic amino acid. Because there are only 20 amino acids encoded in a gene, substitutions that result in a non-functional gene product may be determined by routine experimentation, incorporating amino acids of a different class in the region of the gene product targeted
30 for mutation.

Preferred mutations are in a region of the nucleic acid having an amino acid sequence motif that is conserved among delta-12 fatty acid desaturases or delta-15 fatty acid

desaturases, such as a His-Xaa-Xaa-Xaa-His motif (Tables 1-3). An example of a suitable region has a conserved HECGH motif that is found, for example, in nucleotides corresponding to amino acids 105 to 109 of the *Arabidopsis* and *Brassica* delta-12 desaturase sequences, in nucleotides corresponding to amino acids 101 to 105 of the soybean delta-12 desaturase sequence and in nucleotides corresponding to amino acids 111 to 115 of the maize delta-12 desaturase sequence.^{Table 1} See e.g., WO 94/115116; Okuley et al., Plant Cell 6:147-158 (1994). The one letter amino acid designations used herein are described in Alberts, B. et al., Molecular Biology of the Cell, 3rd edition, Garland Publishing, New York, 1994. Amino acids flanking this motif are also highly conserved among delta-12 and delta-15 desaturases and are also suitable candidates for mutations in fragments of the invention. An illustrative embodiment of a mutation in a nucleic acid fragment of the invention is a Glu to Lys substitution in the HECGH motif of a *Brassica* microsomal delta-12 desaturase sequence, either the D form or the F form. This mutation results in the sequence HECGH being changed to HKGH as seen by comparing SEQ ID NO:¹ (wild-type D form) to SEQ ID NO:² (mutant D form).

A similar motif may be found at amino acids 101 to 105 of the *Arabidopsis* microsomal delta-15 fatty acid desaturase, as well as in the corresponding rape and soybean desaturases (Table 5). See, e.g., WO 93/11245; Arondel, V. et al., Science, 258:1153-1155 (1992); Yadav, N. et al., Plant Physiol., 103:467-476 (1993). Plastid delta-15 fatty acids have a similar motif (Table 5).

Among the types of mutations in an HECGH motif that render the resulting gene product non-functional are non-conservative substitutions. An illustrative example of a non-conservative substitution is substitution of a glycine

residue for either the first or second histidine. Such a substitution replaces a polar residue (histidine) with a non-polar residue (glycine). Another type of mutation that renders the resulting gene product non-functional is an 5 insertion mutation, e.g., insertion of a glycine between the cystine and glutamic acid residues in the HECGH motif.

Other regions having suitable conserved amino acid motifs include the HRRHH motif shown in Table 2 and the HVAHH motif shown in Table 3. See, e.g., WO 94/11516; 10 Hitz, W. et al., Plant Physiol., 105:635-641 (1994); Okuley, J., et al., ^{Supra} and Yaday, N. et al., ^{Supra} ~~Plant Cell, 6:147-158 (1994)~~.

Another region suitable for a mutation in a delta-12 desaturase sequence contains the motif KYLNNP at nucleotides corresponding to amino acids 171 to 175 of the *Brassica* 15 desaturase sequence. An illustrative example of a mutation is this region is a Leu to His substitution, resulting in the amino acid sequence KYHNN. ^(Table 4)

TABLE 1

Alignment of Amino Acid Sequences from Microsomal
20 Delta-12 Fatty Acid Desaturases

<u>Species</u>	<u>Position</u>	<u>Amino Acid Sequence</u>
<i>Arabidopsis thaliana</i>	100-129	IWVIAHECGH HAFSDYQWLD DTVGLIFHSF
<i>Glycine max</i>	96-125	VWVIAHECGH HAFSKYQWVD DVVGLTLHST
<i>Zea mays</i>	106-135	VWVIAHECGH HAFSDYSLLD DVVGLVLHSS
<i>Ricinus communis</i> ^a	1- 29	WVMADCGH HAFSDYQLLD DVVGLILHSC
<i>Brassica napus D</i>	100-128	VWVIAHECGH HAFSDYQWLD DTVGLIFHS
<i>Brassica napus F</i>	100-128	VWVIAHECGH HAFSDYQWLD DTVGLIFHS

^a from plasmid pRF2-1C

TABLE 2

Alignment of Amino Acid Sequences from Microsomal
30 Delta-12 Fatty Acid Desaturases

<u>Species</u>	<u>Position</u>	<u>Amino Acid Sequence</u>
<i>Arabidopsis thaliana</i>	130-158	LLVPYFSWKY SHRRHHSNTG SLERDEVFV
<i>Glycine max</i>	126-154	LLVPYFSWKI SHRRHHSNTG SLDRDEVFV
<i>Zea mays</i>	136-164	LMVPYFSWKY SHRRHHSNTG SLERDEVFV

<i>Ricinus communis</i> ^a	30- 58	LLVPYFSWKH SHRRHHSNTG SLERDEVFV
<i>Brassica napus D</i>	130-158	LLVPYFSWKY SHRSHHSNTG SLERDEVFV
<i>Brassica napus F</i>	130-158	LLVPYFSWKY SHRRHHSNTG SLERDEVFV

^a from plasmid pRF2-1C

TABLE 3

Alignment of Amino Acid Sequences from Microsomal
Delta-12 Fatty Acid Desaturases

	<u>Species</u>	<u>Position</u>	<u>Amino Acid Sequence</u>
5	<i>Arabidopsis thaliana</i>	298-333	DRDYGILNKV FHNIITDTHVA HHLFSTMPHY NAMEAT
	<i>Glycine max</i>	294-329	DRDYGILNKV FHHITDTHVA HHLFSTMPHY HAMEAT
	<i>Zea mays</i>	305-340	DRDYGILNRV FHNIITDTHVA HHLFSTMPHY HAMEAT
	<i>Ricinus communis^a</i>	198-224	DRDYGILNKV FHNIITDTQVA HHLF TMP
	<i>Brassica napus D</i>	299-334	DRDYGILNKV FHNIITDTHVA HHPFSTMPHY HAMEAT
10	<i>Brassica napus F</i>	299-334	DRDYGILNKV FHNIITDTHVA HHLFSTMPHY HAMEAT

^a from plasmid pRF2-1C

TABLE 4

Alignment of Conserved Amino Acids from Microsomal
Delta-12 Fatty Acid Desaturases

	<u>Species</u>	<u>Position</u>	<u>Amino Acid Sequence</u>
15	<i>Arabidopsis thaliana</i>	165-180	IKWYGKYLNN PLGRIM
	<i>Glycine max</i>	161-176	VAWFSLYLN PLGRAV
	<i>Zea mays</i>	172-187	PWYTPYVYNN PVGRVV
	<i>Ricinus communis^a</i>	65- 80	IRWYSKYLNN PPGRIM
20	<i>Brassica napus D</i>	165-180	IKWYGKYLNN PLGRTV
	<i>Brassica napus F</i>	165-180	IKWYGKYLNN PLGRTV

^a from plasmid pRF2-1C

TABLE 5

Alignment of Conserved Amino Acids from Plastid and Microsomal
Delta-15 Fatty Acid Desaturases

	<u>Species</u>	<u>Position</u>	<u>Amino Acid Sequence</u>
25	<i>Arabidopsis thaliana^a</i>	156-177	WALFVLGHD CGHGSFSNDP KLN
	<i>Brassica napus^a</i>	114-135	WALFVLGHD CGHGSFSNDP RLN
	<i>Glycine max^a</i>	164-185	WALFVLGHD CGHGSFSNNS KLN
30	<i>Arabidopsis thaliana</i>	94-115	WAIFVLGHD CGHGSFSDIP LLN
	<i>Brassica napus</i>	85-106	WAIFVLGHD CGHGSFSDIP LLN
	<i>Glycine max</i>	93-114	WALFVLGHD CGHGSFSDSP PLN

^a Plastid sequences

The conservation of amino acid motifs and their relative positions indicates that regions of a delta-12 or delta-15 fatty acid desaturase that can be mutated in one species to generate a non-functional desaturase can be 5 mutated in the corresponding region from other species to generate a non-functional 12-DES or 15-DES gene product in that species.

Mutations in any of the regions of Tables 1-~~8~~ are specifically included within the scope of the invention, 10 provided that such mutation (or mutations) renders the resulting desaturase gene product non-functional, as discussed hereinabove.

A nucleic acid fragment containing a mutant sequence can be generated by techniques known to the skilled artisan. 15 Such techniques include, without limitation, site-directed mutagenesis of wild-type sequences and direct synthesis using automated DNA synthesizers.

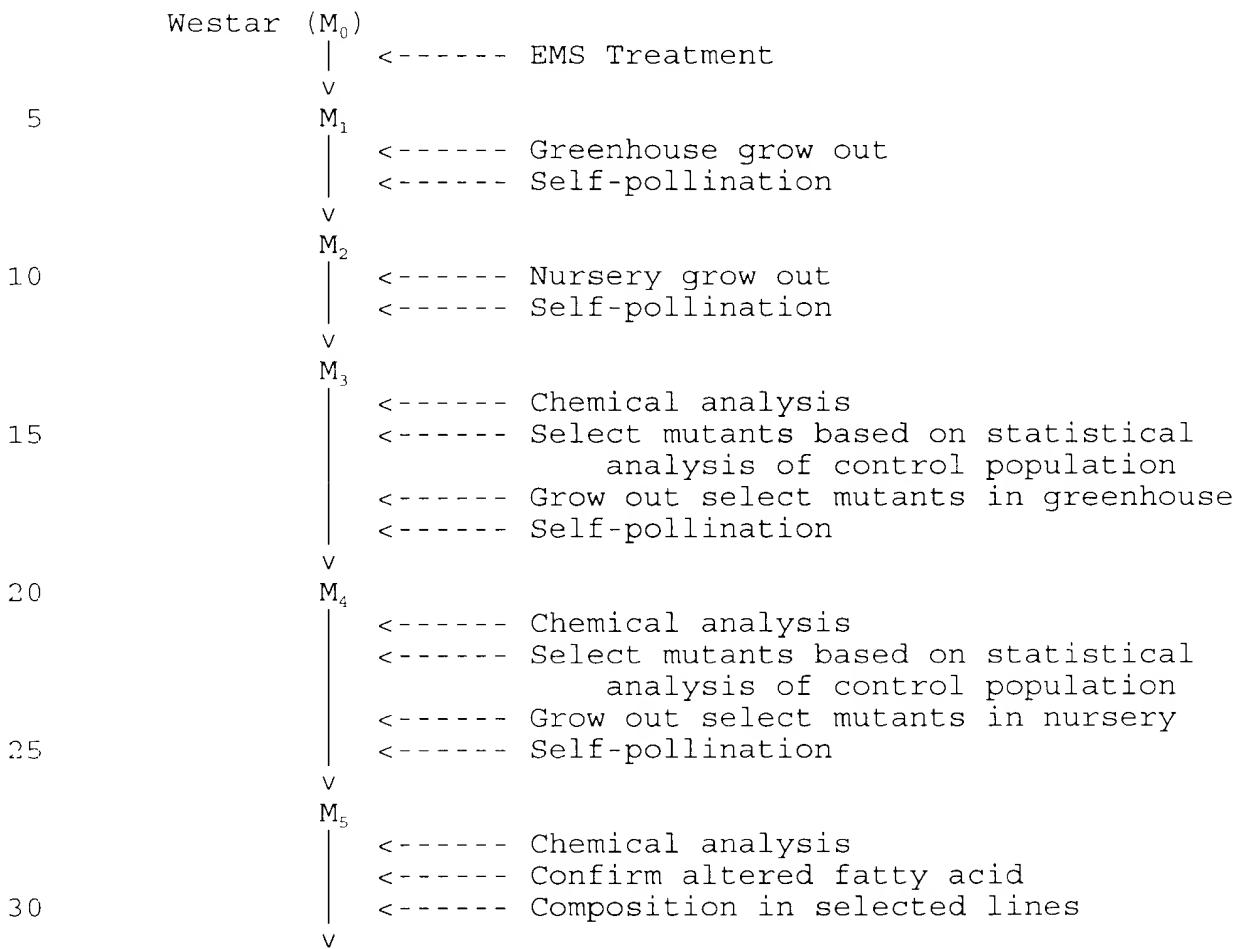
A nucleic acid fragment containing a mutant sequence can also be generated by mutagenesis of plant seeds or 20 regenerable plant tissue by, e.g., ethyl methane sulfonate, X-rays or other mutagens. With mutagenesis, mutant plants having the desired fatty acid phenotype in seeds are identified by known techniques and a nucleic acid fragment containing the desired mutation is isolated from genomic DNA 25 or RNA of the mutant line. The site of the specific mutation is then determined by sequencing the coding region of the 12-DES or 15-DES gene. Alternatively, labeled nucleic acid probes that are specific for desired mutational events can be used to rapidly screen a mutagenized 30 population.

Seeds of Westar, a Canadian (*Brassica napus*) spring canola variety, were ^{subjected} ~~subject~~ to chemical mutagenesis. Mutagenized seeds were planted in the greenhouse and the

plants were self-pollinated. The progeny plants were individually analyzed for fatty acid composition, and regrown either in the greenhouse or in the field. After four successive generations of self-pollinations, followed
5 by chemical analysis of the seed oil at each cycle, several lines were shown to carry stably inherited mutations in specific fatty acid components, including reduced palmitic acid ($C_{16:0}$), increased palmitic acid, reduced stearic acid ($C_{18:0}$), increased oleic acid ($C_{18:1}$), reduced linoleic acid
10 ($C_{18:2}$) and reduced linolenic acid ($C_{18:3}$), in the seed oil.

The general experimental scheme for developing lines with stable fatty acid mutations is shown in Scheme I hereinafter.

SCHEME I



STABLE FATTY ACID MUTANTS

Westar seeds (M_0) were mutagenized with ethylmethanesulfonate (EMS). Westar is a registered 35 Canadian spring variety with canola quality. The fatty acid composition of field-grown Westar, 3.9% $C_{16:0}$, 1.9% $C_{18:0}$, 67.5% $C_{18:1}$, 17.6% $C_{18:2}$, 7.4% $C_{18:3}$, <2% $C20:1 + C22:1$, has remained stable under commercial production, with $\pm 10\%$ deviation, since 1982. The disclosed method may be applied 40 to all oilseed *Brassica* species, and to both Spring and Winter maturing types within each species. Physical mutagens, including but not limited to X-rays, UV rays, and

other physical treatments which cause chromosome damage, and other chemical mutagens, including but not limited to ethidium bromide, nitrosoguanidine, diepoxybutane etc. may also be used to induce mutations. The mutagenesis treatment 5 may also be applied to other stages of plant development, including but not limited to cell cultures, embryos, microspores and shoot apices. The M₁ seeds were planted in the greenhouse and M₁ plants were individually self-pollinated.

10 M₂ seed was harvested from the greenhouse and planted in the field in a plant-to-row design. Each plot contained six rows, and five M₂ lines were planted in each plot. Every other plot contained a row of non-mutagenized Westar as a control. Based on gas chromatographic analysis 15 of M₂ seed, those lines which had altered fatty acid composition were self-pollinated and individually harvested.

M₃ seeds were evaluated for mutations on the basis of a Z-distribution. An extremely stringent 1 in 10,000 rejection rate was employed to establish statistical 20 thresholds to distinguish mutation events from existing variation. Mean and standard deviation values were determined from the non-mutagenized Westar control population in the field. The upper and lower statistical thresholds for each fatty acid were determined from the mean 25 value of the population \pm the standard deviation, multiplied by the Z-distribution. Based on a population size of 10,000, the confidence interval is 99.99%.

Seeds (M₃) from those M₂ lines which exceeded either 30 the upper or lower statistical thresholds were replanted in the greenhouse and self-pollinated. This planting also included Westar controls. The M₄ seed was re-analyzed using new statistical thresholds established with a new control population. Those M₄ lines which exceeded the new

statistical thresholds for selected fatty acid compositions were advanced to the nursery. Following self-pollination, M₅ seed from the field were re-analyzed once again for fatty acid composition. Those lines which remained stable for the 5 selected fatty acids were considered stable mutations.

"Stable mutations" as used herein are defined as M₅ or more advanced lines which maintain a selected altered fatty acid profile for a minimum of three generations, including a minimum of two generations under field 10 conditions, and exceeding established statistical thresholds for a minimum of two generations, as determined by gas chromatographic analysis of a minimum of 10 randomly selected seeds bulked together. Alternatively, stability may be measured in the same way by comparing to subsequent 15 generations. In subsequent generations, stability is defined as having similar fatty acid profiles in the seed as that of the prior or subsequent generation when grown under substantially similar conditions.

The amount of variability for fatty acid content in 20 a seed population is quite significant when single seeds are analyzed. Randomly selected single seeds and a ten seed bulk sample of a commercial variety were compared. Significant variation among the single seeds was detected (Table A). The half-seed technique (Downey, R.K. and B.L. 25 Harvey, Can. J. Plant Sci., 43:271 [1963]) in which one cotyledon of the germinating seed is analyzed for fatty acid composition and the remaining embryo grown into a plant has been very useful to plant breeding work to select individuals in a population for further generation analysis. 30 The large variation seen in the single seed analysis (Table A) is reflected in the half-seed technique.

TABLE A

Single Seed Analysis for Fatty Acid Composition¹

SAMPLE	16:0	16:1	18:0	18:1	18:2	18:3	20:0	20:1	22:0	22:1
Bulk	3.2	0.4	1.8	20.7	13.7	9.8	0.8	11.2	0.4	32.2
5 1	2.8	0.2	1.1	14.6	14.6	11.1	0.8	9.8	0.7	38.2
2	3.3	0.2	1.3	13.1	14.4	11.7	0.9	10.5	0.7	37.0
3	3.0	--	1.2	12.7	15.3	10.6	0.8	7.3	0.7	43.2
4	2.8	0.2	1.1	16.7	13.2	9.1	0.8	11.2	0.4	38.9
5	3.0	--	1.8	15.2	13.3	8.4	1.3	8.7	0.9	42.3
10 6	3.1	--	1.3	14.4	14.6	10.3	1.0	10.9	0.8	39.3
7	2.6	--	1.2	15.7	13.8	9.9	0.9	12.2	0.5	37.0
8	3.1	--	1.1	16.2	13.4	10.6	0.6	9.2	0.8	41.4
9	2.7	0.1	1.0	13.5	11.2	11.3	0.8	6.2	0.7	46.9
10	3.4	0.2	1.4	13.9	17.5	10.8	1.1	10.0	0.9	36.2
15 11	2.8	0.2	1.2	12.7	12.9	10.3	1.0	7.9	0.9	43.3
12	2.3	0.1	1.6	20.7	14.8	6.5	1.1	12.5	0.8	34.5
13	2.6	0.2	1.3	21.0	11.4	7.6	1.0	11.6	0.6	36.7
14	2.6	0.1	1.2	14.7	13.2	9.4	0.9	10.1	0.8	40.8
15	2.9	0.2	1.4	16.6	15.1	11.2	0.7	9.1	0.3	36.1
20 16	3.0	0.2	1.1	12.4	13.7	10.4	0.9	8.7	0.8	42.7
17	2.9	0.1	1.1	21.1	12.3	7.1	0.8	12.4	0.5	36.8
18	3.1	0.1	1.2	13.7	13.1	10.4	1.0	8.8	0.7	41.6
19	2.7	0.1	1.0	11.1	13.4	11.7	0.8	7.9	0.8	43.5
20	2.3	0.2	0.2	18.2	13.9	8.2	0.9	10.3	0.8	38.2
25Average	2.8	0.2	1.2	15.4	13.8	9.8	0.9	9.8	0.7	39.7
Minimum	2.3	0.1	0.2	11.1	11.2	6.5	0.6	6.2	0.3	34.5
Maximum	3.4	0.2	1.8	21.1	17.5	11.7	1.3	12.5	0.9	46.9
Range	1.1	0.1	1.6	9.9	6.3	5.3	0.7	6.4	0.6	12.4

¹Values expressed as percent of total oil

30 Plant breeders using the half-seed technique have found it unreliable in selecting stable genetically controlled fatty acid mutations (Stefanson, B.R., In; High

and Low Erucic Acid Rapeseed Oils, Ed. N.T. Kenthies, Academic Press, Inc., Canada (1983) pp. 145-159). Although valuable in selecting individuals from a population, the selected traits are not always transmitted to subsequent 5 generations (Rakow, G. and McGregor, D.I., J. Amer. Oil Chem. Soc. (1973) 50:400-403. To determine the genetic stability of the selected plants several self-pollinated generations are required (Robelen, G. In: Biotechnology for the Oils and Fats Industry, Ed. C. Ratledge, P. Dawson and 10 J. Rattray, American Oil Chemists Society (1984) pp. 97-105) with chemical analysis of a bulk seed sample.

Mutation breeding has traditionally produced plants carrying, in addition to the trait of interest, multiple, deleterious traits, e.g., reduced plant vigor and reduced 15 fertility. Such traits may indirectly affect fatty acid composition, producing an unstable mutation; and/or reduce yield, thereby reducing the commercial utility of the invention. To eliminate the occurrence of deleterious mutations and reduce the load of mutations carried by the 20 plant a low mutagen dose was used in the seed treatments to create an LD30 population. This allowed for the rapid selection of single gene mutations for fatty acid traits in agronomic backgrounds which produce acceptable yields.

Other than changes in the fatty acid composition of 25 the seed oil, the mutant lines described here have normal plant phenotype when grown under field conditions, and are commercially useful. "Commercial utility" is defined as having a yield, as measured by total pounds of seed or oil produced per acre, within 15% of the average yield of the 30 starting (M_0) canola variety grown in the same region. To be commercially useful, plant vigor and high fertility are such that the crop can be produced in this yield by farmers using conventional farming equipment, and the oil with

altered fatty acid composition can be extracted using conventional crushing and extraction equipment.

The seeds of several different fatty acid lines have been deposited with the American Type Culture Collection and
5 have the following accession numbers.

	<u>Line</u>	<u>Accession No.</u>	<u>Deposit Date</u>
	A129.5	40811	May 25, 1990
	A133.1	40812	May 25, 1990
	A144.1	40813	May 25, 1990
10	A200.7	40816	May 31, 1990
	M3032.1	75021	June 7, 1991
	M3094.4	75023	June 7, 1991
	M3052.6	75024	June 7, 1991
	M3007.4	75022	June 7, 1991
15	M3062.8	75025	June 7, 1991
	M3028.10	75026	June 7, 1991
	IMC130	75446	April 16, 1993

In some plant species or varieties more than one form of endogenous microsomal delta-12 desaturase may be
20 found. In amphidiploids, each form may be derived from one of the parent genomes making up the species under consideration. Plants with mutations in both forms have a fatty acid profile that differs from plants with a mutation in only one form. An example of such a plant is *Brassica napus* line Q508, a doubly-mutagenized line containing a mutant ^FR-form of delta-12 desaturase (SEQ ID NO:1) and a mutant ^FR-form of delta-12 desaturase (SEQ ID NO:5).

Preferred host or recipient organisms for introduction of a nucleic acid fragment of the invention are
30 the oil-producing species, such as soybean (*Glycine max*), rapeseed (e.g., *Brassica napus*, *B. rapa* and *B. juncea*), sunflower (*Helianthus annus*), castor bean (*Ricinus communis*), corn (*Zea mays*), and safflower (*Carthamus tinctorius*).

Plants according to the invention preferably contain an altered fatty acid profile. For example, oil obtained from seeds of such plants may have from about 69 to about 90% oleic acid, based on the total fatty acid composition of 5 the seed. Such oil preferably has from about 74 to about 90% oleic acid, more preferably from about 80 to about 90% oleic acid. In some embodiments, oil obtained from seeds produced by plants of the invention may have from about 2.0% to about 5.0% saturated fatty acids, based on total fatty 10 acid composition of the seeds. In some embodiments, oil obtained from seeds of the invention may be from about 1.0% to about 10.0% linoleic acid, or from about 0.5% to about 10.0% α -linolenic acid.

In one embodiment of the claimed invention, a plant 15 contains both a 12-DES mutation and a 15-DES mutation. Such plants can have a fatty acid composition comprising very high oleic acid and very low alpha-linolenic acid levels. Mutations in 12-DES and 15-DES may be combined in a plant by making a genetic cross between 12-DES and 15-DES single 20 mutant lines. A plant having a mutation in delta-12 fatty acid desaturase is crossed or mated with a second plant having a mutation in delta-15 fatty acid desaturase. Seeds produced from the cross are planted and the resulting plants are selfed in order to obtain progeny seeds. These progeny 25 seeds are then screened in order to identify those seeds carrying both mutant genes.

Alternatively, a line possessing either a 12-DES or a 15-DES mutation can be subjected to mutagenesis to generate a plant or plant line having mutations in both 12-30 DES and 15-DES. For example, the IMC129 line has a mutation in the coding region (Glu₁₀₆ to Lys₁₀₆) of the D form of the microsomal delta-12 desaturase structural gene. Cells (e.g., seeds) of this line can be mutagenized to induce a

mutation in a 15-DES gene, resulting in a plant or plant line carrying a mutation in a delta-12 fatty acid desaturase gene and a mutation in a delta-15 fatty acid desaturase gene.

5 Progeny includes descendants of a particular plant or plant line, e.g., seeds developed on an instant plant. Progeny of an instant plant include seeds formed on F_1 , F_2 , F_3 , and subsequent generation plants, or seeds formed on BC_1 , BC_2 , BC_3 and subsequent generation plants.

10 Those seeds having an altered fatty acid composition may be identified by techniques known to the skilled artisan, e.g., gas-liquid chromatography (GLC) analysis of a bulked seed sample or of a single half-seed. Half-seed analysis is well known in the art to be useful because the 15 viability of the embryo is maintained and thus those seeds having a desired fatty acid profile may be planted to from the next generation. However, half-seed analysis is also known to be an inaccurate representation of genotype of the seed being analyzed. Bulk seed analysis typically yields a 20 more accurate representation of the fatty acid profile of a given genotype.

Line 24
The nucleic acid fragments of the invention can be used as markers in plant genetic mapping and plant breeding programs. Such markers may include RFLP, RAPD, or PCR 25 markers, for example. Marker-assisted breeding techniques may be used to identify and follow a desired fatty acid composition during the breeding process. Marker-assisted breeding techniques may be used in addition to, or as an alternative to, other sorts of identification techniques.
30 An example of marker-assisted breeding is the use of PCR primers that specifically amplify a sequence containing a desired mutation in 12-DES or 15-DES.

Methods according to the invention are useful in that the resulting plants and plant lines have desirable seed fatty acid compositions as well as superior agronomic properties compared to known lines having altered seed fatty acid composition. Superior agronomic characteristics include, for example, increased seed germination percentage, increased seedling vigor, increased resistance to seedling fungal diseases (damping off, root rot and the like), increased yield, and improved standability.

While the invention is susceptible to various modifications and alternative forms, certain specific embodiments thereof are described in the general methods and examples set forth below. For example the invention may be applied to all *Brassica* species, including *B. rapa*, *B. juncea*, and *B. hirta*, to produce substantially similar results. It should be understood, however, that these examples are not intended to limit the invention to the particular forms disclosed but, instead the invention is to cover all modifications, equivalents and alternatives falling within the scope of the invention. This includes the use of somaclonal variation; physical or chemical mutagenesis of plant parts; anther, microspore or ovary culture followed by chromosome doubling; or self- or cross-pollination to transmit the fatty acid trait, alone or in combination with other traits, to develop new *Brassica* lines.

EXAMPLE 1

Selection of Low FDA Saturates

Prior to mutagenesis, 30,000 seeds of *B. napus* cv. Westar seeds were preimbibed in 300-seed lots for two hours on wet filter paper to soften the seed coat. The preimbibed seeds were placed in 80 mM ethylmethanesulfonate (EMS) for

four hours. Following mutagenesis, the seeds were rinsed three times in distilled water. The seeds were sown in 48-well flats containing Pro-Mix. Sixty-eight percent of the mutagenized seed germinated. The plants were maintained at 5 25°C/15°C, 14/10 hr day/night conditions in the greenhouse. At flowering, each plant was individually self-pollinated.

M₂ seed from individual plants were individually catalogued and stored, approximately 15,000 M₂ lines was planted in a summer nursery in Carman, Manitoba. The seed 10 from each selfed plant were planted in 3-meter rows with 6-inch row spacing. Westar was planted as the check variety. Selected lines in the field were selfed by bagging the main raceme of each plant. At maturity, the selfed plants were individually harvested and seeds were catalogued and stored 15 to ensure that the source of the seed was known.

Self-pollinated M₃ seed and Westar controls were analyzed in 10-seed bulk samples for fatty acid composition via gas chromatography. Statistical thresholds for each fatty acid component were established using a Z-distribution 20 with a stringency level of 1 in 10,000. The selected M₃ seeds were planted in the greenhouse along with Westar controls. The seed was sown in 4-inch pots containing Pro-Mix soil and the plants were maintained at 25°C/15°C, 14/10 hr day/night cycle in the greenhouse. At flowering, the 25 terminal raceme was self-pollinated by bagging. At maturity, selfed M₄ seed was individually harvested from each plant, labelled, and stored to ensure that the source of the seed was known.

The M₄ seed was analyzed in 10-seed bulk samples. 30 Statistical thresholds for each fatty acid component were established from 259 control samples using a Z-distribution of 1 in 800. Selected M₄ lines were planted in a field trial in Carman, Manitoba in 3-meter rows with 6-inch

spacing. Ten M₄ plants in each row were bagged for self-pollination. At maturity, the selfed plants were individually harvested and the open pollinated plants in the row were bulk harvested. The M₅ seed from single plant 5 selections was analyzed in 10-seed bulk samples and the bulk row harvest in 50-seed bulk samples.

Selected M₅ lines were planted in the greenhouse along with Westar controls. The seed was grown as previously described. At flowering the terminal raceme was 10 self-pollinated by bagging. At maturity, selfed M₆ seed was individually harvested from each plant and analyzed in 10-seed bulk samples for fatty acid composition.

Selected M₆ lines were entered into field trials in Eastern Idaho. The four trial locations were selected for 15 the wide variability in growing conditions. The locations included Burley, Teton, Lamont and Shelley (Table I). The lines were planted in four 3-meter rows with an 8-inch spacing, each plot was replicated four times. The planting design was determined using a Randomized Complete Block 20 Designed. The commercial cultivar Westar was used as a check cultivar. At maturity the plots were harvested to determine yield. Yield of the entries in the trial was determined by taking the statistical average of the four replications. The Least Significant Difference Test was 25 used to rank the entries in the randomized complete block design.

TABLE I
Trial Locations for Selected Fatty Acid Mutants

LOCATION	SITE CHARACTERIZATIONS
BURLEY	Irrigated. Long season. High temperatures during flowering.
TETONIA	Dryland. Short season. Cool temperatures.
LAMONT	Dryland. Short season. Cool temperatures.
SHELLEY	Irrigated. Medium season. High temperatures during flowering.

10 To determine the fatty acid profile of entries, plants in each plot were bagged for self-pollination. The M₁ seed from single plants was analyzed for fatty acids in ten-seed bulk samples.

15 To determine the genetic relationships of the selected fatty acid mutants crosses were made. Flowers of M₆ or later generation mutations were used in crossing. F₁ seed was harvested and analyzed for fatty acid composition to determine the mode of gene action. The F₁ progeny were planted in the greenhouse. The resulting plants were self-pollinated, the F₂ seed harvested and analyzed for fatty acid composition for allelism studies. The F₂ seed and parent line seed was planted in the greenhouse, individual plants were self-pollinated. The F₃ seed of individual plants was tested for fatty acid composition using 10-seed 20 bulk samples as described previously.

25 In the analysis of some genetic relationships dihaploid populations were made from the microspores of the F₁ hybrids. Self-pollinated seed from dihaploid plants were analyzed for fatty acid analysis using methods described 30 previously.

For chemical analysis, 10-seed bulk samples were hand ground with a glass rod in a 15-mL polypropylene tube and extracted in 1.2 mL 0.25 N KOH in 1:1 ether/methanol. The sample was vortexed for 30 sec. and heated for 60 sec. 5 in a 60°C water bath. Four mL of saturated NaCl and 2.4 mL of iso-octane were added, and the mixture was vortexed again. After phase separation, 600 μ L of the upper organic phase were pipetted into individual vials and stored under nitrogen at -5°C. One μ L samples were injected into a 10 Supelco SP-2330 fused silica capillary column (0.25 mm ID, 30 M length, 0.20 μ m df).

The gas chromatograph was set at 180°C for 5.5 minutes, then programmed for a 2°C/minute increase to 212°C, and held at this temperature for 1.5 minutes. Total run 15 time was 23 minutes. Chromatography settings were: Column head pressure - 15 psi, Column flow (He) - 0.7 mL/min., Auxiliary and Column flow - 33 mL/min., Hydrogen flow - 33 mL/min., Air flow - 400 mL/min., Injector temperature - 250°C, Detector temperature - 300°C, Split vent - 1/15.

20 Table II describes the upper and lower statistical thresholds for each fatty acid of interest.

TABLE II
 Statistical Thresholds for Specific Fatty Acids
Derived from Control Westar Plantings

5	Genotype	Percent Fatty Acids					Sats*
		C _{16:0}	C _{18:0}	C _{18:1}	C _{18:2}	C _{18:3}	
M₃ Generation (1 in 10,000 rejection rate)							
	Lower	3.3	1.4	--	13.2	5.3	6.0
	Upper	4.3	2.5	71.0	21.6	9.9	8.3
M₄ Generation (1 in 800 rejection rate)							
10	Lower	3.6	0.8	--	12.2	3.2	5.3
	Upper	6.3	3.1	76.0	32.4	9.9	11.2
M₅ Generation (1 in 755 rejection rate)							
	Lower	2.7	0.9	--	9.6	2.6	4.5
15	Upper	5.7	2.7	80.3	26.7	9.6	10.0

*Sats=Total Saturate Content

At the M₃ generation, twelve lines exceeded the lower statistical threshold for palmitic acid ($\leq 3.3\%$). Line W13097.4 had 3.1% palmitic acid and an FDA saturate content of 4.5%. After a cycle in the greenhouse, M₄ seed from line W13097.4 (designated line A144) was analyzed. Line W13097.4.1 (A144.1) had 3.1% C_{16:0}, exceeding the lower statistical threshold of 3.6%. The FDA saturate content for A144.1 was 4.5%. The fatty acid compositions for the M₃, M₄ and M₅ generations of this family are summarized in Table III.

TABLE III

Fatty Acid Composition of a Low Palmitic Acid/Low FDA Saturate Canola Line Produced by Seed Mutagenesis

5	Genotype ^a	Percent Fatty Acids						
		C _{16:0}	C _{18:0}	C _{18:1}	C _{18:2}	C _{18:3}	Sats ^b	Tot Sat ^c
	Westar	3.9	1.9	67.5	17.6	7.4	5.9	7.0
	W13097.4 (M ₃)	3.1	1.4	63.9	18.6	9.5	4.5	5.6
10	W13097.4 (M ₄)	3.1	1.4	66.2	19.9	6.0	4.5	5.5
	A144.1.9 (M ₅)	2.9	1.4	64.3	20.7	7.3	4.4	5.3

15 ^aLetter and numbers up to second decimal point indicate the plant line. Number after second decimal point indicates an individual plant.

^bSat=FDA Saturates

^cTot Sat=Total Saturate Content

20 The M₅ seed of ten self-pollinated A144.1 (ATCC 40813) plants averaged 3.1% palmitic acid and 4.7% FDA saturates. One selfed plant (A144.1.9) contained 2.9% palmitic acid and FDA saturates of 4.4%. Bulk seed analysis from open-pollinated (A144.1) plants at the M₅ generation averaged 3.1% palmitic acid and 4.7% FDA saturates. The fatty acid composition of the bulked and individual A144.1 lines are summarized in Table IV.

TABLE IV
 Fatty Acid Composition of A144
Low Palmitic Acid/Low FDA Saturate Line

5	Genotype ^a	Percent Fatty Acids						
		C _{16:0}	C _{18:0}	C _{18:1}	C _{18:2}	C _{18:3}	Sats ^b	Tot Sat ^c
Individually Self-Pollinated Plants								
	A144.1.1	3.2	1.6	64.4	20.5	7.0	4.8	5.9
	A144.1.2	3.0	1.5	67.4	18.6	6.3	4.5	5.7
10	A144.1.3	3.6	1.8	61.4	22.4	7.5	5.2	6.6
	A144.1.4	3.2	1.5	64.6	20.9	6.7	4.7	5.8
	A144.1.5	3.3	1.7	60.0	23.9	7.9	5.0	6.1
	A144.1.6	3.1	1.4	67.3	17.8	6.5	4.6	5.2
	A144.1.7	3.1	1.6	67.7	17.4	6.5	4.8	5.4
15	A144.1.8	3.1	1.8	66.9	18.7	6.1	4.9	5.4
	A144.1.9	2.9	1.4	64.3	20.7	7.3	4.4	5.3
	A144.1.10	3.1	1.5	62.5	20.4	7.7	4.6	5.6
Average of Individually Self-Pollinated Plants								
	A144.1.1-10	3.1	1.6	64.8	20.1	6.9	4.7	5.7
20	Bulk Analysis of Open-Pollinated Plants							
	A144.1B	3.1	1.6	64.8	19.4	7.8	4.7	5.7

^aLetter and numbers up to second decimal point indicate the plant line. Number after second decimal point indicates an individual plant.

^bSat=FDA Saturates

^cTot Sat=Total Saturate Content

These reduced levels have remained stable to the M₇ generations in both greenhouse and field conditions. These 30 reduced levels have remained stable to the M₇ generation in multiple location field trials. Over all locations, the self-pollinated plants (A144) averaged 2.9% palmitic acid

and FDA saturates of 4.6%. The fatty acid composition of the A144 lines for each Idaho location are summarized in Table V. In the multiple location replicated trial the yield of A144 was not significantly different in yield from 5 the parent cultivar Westar. By means of seed mutagenesis, the level of saturated fatty acids of canola (*B. napus*) was reduced from 5.9% to 4.6%. The palmitic acid content was reduced from 3.9% to 2.9%.

TABLE V

10 Fatty Acid Composition of a Mutant Low Palmitic Acid/Low FDA Saturate Canola Line at Different Field Locations in Idaho

15	Trial Location	Percent Fatty Acids						
		C _{16:0}	C _{18:0}	C _{18:1}	C _{18:2}	C _{18:3}	Sats	Tot
	Burley	2.9	1.3	62.3	20.6	10.3	4.2	5.0
	Tetonia	2.9	1.7	59.7	21.0	11.2	4.6	5.7
	Lamont	3.1	1.8	63.2	19.5	9.0	4.9	5.9
20	Shelley	2.8	1.9	64.5	18.8	8.8	4.7	5.9

To determine the genetic relationship of the palmitic acid mutation in A144 (C_{16:0} - 3.0%, C_{18:0} - 1.5%, C_{18:1} - 67.4%, C_{18:2} - 18.6%, C_{18:3} - 6.3%) to other fatty acid mutations it was crossed to A129 a mutant high oleic acid (C_{16:0} - 3.8%, C_{18:0} - 2.3%, C_{18:1} - 75.6%, C_{18:2} - 9.5%, C_{18:3} - 4.9%). Over 570 dihaploid progeny produced from the F₁ hybrid were harvested and analyzed for fatty acid composition. The results of the progeny analysis are 25 summarized in Table VB. Independent segregation of the palmitic traits was observed which demonstrates that the 30

genetic control of palmitic acid in A144 is different from the high oleic acid mutation in A129.

TABLE VB

Genetic Studies of Dihaploid Progeny of A144 X A129

5	Genotype	C _{16:0} Content (%)	Frequency	
			Observed	Expected
	p-p-p2-p2-	3.0%	162	143
10	p+p+p2-p2-	3.4%	236	286
	p+p+p2+p2+	3.8%	175	143

EXAMPLE 2

An additional low FDA saturate line, designated A149.3 (ATCC 40814), was also produced by the method of Example 1. A 50-seed bulk analysis of this line showed the following fatty acid composition: C_{16:0} - 3.6%, C_{18:0} - 1.4%, C_{18:1} - 65.5%, C_{18:2} - 18.3%, C_{18:3} - 8.2%, FDA Sats - 5.0%, Total Sats - 5.9%. This line has also stably maintained its mutant fatty acid composition to the M₅ generation. In a multiple location replicated trial the yield of A149 was not significantly different in yield from the parent cultivar Westar.

EXAMPLE 3

An additional low palmitic acid and low FDA saturate line, designated M3094.4 (ATCC 75023), was also produced by the method of Example 1. A 10-seed bulk analysis of this line showed the following fatty acid composition: C_{16:0} - 2.7%, C_{18:0} - 1.6%, C_{18:1} - 66.6%, C_{18:2} - 20.0%, C_{18:3} - 6.1%, C_{20:1} - 1.4%, C_{22:1} - 0.0%, FDA Saturate - 4.3%, Total Saturates - 5.2%. This line has stably maintained its mutant fatty acid composition to the M₅ generation. In a

single replicated trial the yield of M3094 was not significantly different in yield from the parent cultivar.

M3094.4 was crossed to A144, a low palmitic acid mutation (Example 1) for allelism studies. Fatty acid 5 composition of the F_2 seed showed the two lines to be allelic. The mutational events in A144 and M3094, although different in origin, are in the same gene.

EXAMPLE 4

In the studies of Example 1, at the M_3 generation, 10 470 lines exceed the upper statistical threshold for palmitic acid ($\geq 4.3\%$). One M_3 line, W14538.6, contained 9.2% palmitic acid. Selfed progenies of this line, since designated M3007.4 (ATCC 75022), continued to exceed to the upper statistical threshold for high palmitic acid at both 15 the M_4 and M_5 generations with palmitic acid levels of 11.7% and 9.1%, respectively. The fatty acid composition of this high palmitic acid mutant, which was stable to the M_7 generation under both field and greenhouse conditions, is summarized in Table VI.

TABLE VI
**Fatty Acid Composition of a High Palmitic
 Acid Canola Line Produced by Seed Mutagenesis**

5	Genotype	Percent Fatty Acids					
		C _{16:0}	C _{18:0}	C _{18:1}	C _{18:2}	C _{18:3}	Sats*
	Westar	3.9	1.9	67.5	17.6	7.4	7.0
	W114538.6 (M ₃)	8.6	1.6	56.4	20.3	9.5	10.2
10	M3007.2 (M ₄)	11.7	2.1	57.2	18.2	5.1	13.9
	M3007.4 (M ₅)	9.1	1.4	63.3	13.7	5.5	12.7

*Sats=Total Saturate Content

15 To determine the genetic relationship of the high palmitic mutation in M3007.4 to the low palmitic mutation in A144 (Example 1) crosses were made. The F₂ progeny were analyzed for fatty acid composition. The data presented in Table VIB shows the high palmitic group (C_{16:0} > 7.0%) makes up one-quarter of the total population analyzed. The high palmitic acid mutation was controlled by one single gene mutation.

TABLE VIB
Genetic Studies of M3007 X A144

25	Genotype	Frequency		
		C _{16:0} Content (%)	Observed	Expected
	p-p-/p-hp-	<7.0	151	142
	hp-hp-	>7.0	39	47

30 An additional M₃ line, W4773.7, contained 4.5% palmitic acid. Selfed progenies of this line, since

designated A200.7 (ATCC 40816), continued to exceed the upper statistical threshold for high palmitic acid in both the M₄ and M₅ generations with palmitic acid levels of 6.3% and 6.0%, respectively. The fatty acid composition of this 5 high palmitic acid mutant, which was stable to the M₇ generation under both field and greenhouse conditions, is summarized in Table VII.

TABLE VII
Fatty Acid Composition of a High Palmitic
10 Acid Canola Line Produced by Seed Mutagenesis

Genotype	Percent Fatty Acids					
	C _{16:0}	C _{18:0}	C _{18:1}	C _{18:2}	C _{18:3}	Sats*
Westar	3.9	1.9	67.5	17.6	7.4	7.0
15 W4773.7 (M ₃)	4.5	2.9	63.5	19.9	7.1	9.3
M4773.7.7 (M ₄)	6.3	2.6	59.3	20.5	5.6	10.8
20 A200.7.7 (M ₅)	6.0	1.9	60.2	20.4	7.3	9.4

*Sats=Total Saturate Content

EXAMPLE 5

Selection of Low Stearic Acid Canola Lines

25 In the studies of Example 1, at the M₃ generation, 42 lines exceeded the lower statistical threshold for stearic acid (<1.4%). Line W14859.6 had 1.3% stearic acid. At the M₅ generation, its selfed progeny (M3052.1) continued to fall within the lower statistical threshold for C_{18:0} with 30 0.8% stearic acid. The fatty acid composition of this low stearic acid mutant, which was stable under both field and

greenhouse conditions is summarized in Table VIII. In a single location replicated yield trial M3052.1 was not significantly different in yield from the parent cultivar Westar.

5

TABLE VIII
Fatty Acid Composition of a Low
Stearic Acid Canola Line Produced by Seed Mutagenesis

Genotype	Percent Fatty Acids					
	C _{16:0}	C _{18:0}	C _{18:1}	C _{18:2}	C _{18:3}	Sats
Westar	3.9	1.9	67.5	17.6	7.4	5.9
W14859.6 (M ₃)	5.3	1.3	56.1	23.7	9.6	7.5
M3052.1 (M ₄)	4.9	0.9	58.9	22.7	9.3	5.8
M3052.6 (M ₅)	4.4	0.8	62.1	21.2	7.9	5.2

To determine the genetic relationship of the low stearic acid mutation of M3052.1 to other fatty acid mutations it was crossed to the low palmitic acid mutation A144 (Example 1). Seed from over 300 dihaploid progeny were harvested and analyzed for fatty acid composition. The results are summarized in Table VIIIB. Independent segregation of the palmitic acid and stearic acid traits was observed. The low stearic acid mutation was genetically different from the low palmitic acid mutations found in A144 and M3094.

TABLE VIIIB
Genetic Studies of M3052 X A144

5	Genotype	$C_{16:0} + C_{18:0}$ Content (%)	Frequency	
			Observed	Expected
	p-p-s-s-	<4.9%	87	77
	p-p-s-s-/p+p+s-s-	4.0% < X < 5.6%	152	154
	p+p+s+s+	>5.6%	70	77

An additional M_5 line, M3051.10, contained 0.9% and
10 1.1% stearic acid in the greenhouse and field respectively.
A ten-seed analysis of this line showed the following fatty
acid composition: $C_{16:0}$ - 3.9%, $C_{18:0}$ - 1.1%, $C_{18:1}$ - 61.7%, $C_{18:2}$
- 23.0%, $C_{18:3}$ - 7.6%, FDA saturates - 5.0%, Total Saturates -
5.8%. In a single location replicated yield trial M3051.10
15 was not significantly different in yield from the parent
cultivar Westar. M3051.10 was crossed to M3052.1 for
allelism studies. Fatty acid composition of the F_2 seed
showed the two lines to be allelic. The mutational events
in M3051.10 and M3052.1 although different in origin were in
20 the same gene.

An additional M_5 line, M3054.7, contained 1.0% and
1.3% stearic acid in the greenhouse and field respectively.
A ten-seed analysis of this line showed the following fatty
acid composition: $C_{16:0}$ - 4.0%, $C_{18:0}$ - 1.0%, $C_{18:1}$ - 66.5%, $C_{18:2}$
- 18:4%, $C_{18:3}$ - 7.2%, saturates - 5.0%, Total Saturates -
6.1%. In a single location replicated yield trial M3054.7
25 was not significantly different in yield from the parent
cultivar Westar. M3054.7 was crossed to M3052.1 for
allelism studies. Fatty acid composition of the F_2 seed
showed the two lines to be allelic. The mutational events
30

in M3054.7, M3051.10 and M3052.1 although different in origin were in the same gene.

EXAMPLE 6

High Oleic Acid Canola Lines

5 In the studies of Example 1, at the M_3 generation, 31 lines exceeded the upper statistical threshold for oleic acid ($\geq 71.0\%$). Line W7608.3 had 71.2% oleic acid. At the M_4 generation, its selfed progeny (W7608.3.5, since designated A129.5) continued to exceed the upper statistical
10 threshold for $C_{18:1}$ with 78.8% oleic acid. M_5 seed of five self-pollinated plants of line A129.5 (ATCC 40811) averaged 75.0% oleic acid. A single plant selection, A129.5.3 had 75.6% oleic acid. The fatty acid composition of this high oleic acid mutant, which was stable under both field and
15 greenhouse conditions to the M_7 generation, is summarized in Table IX. This line also stably maintained its mutant fatty acid composition to the M_7 generation in field trials in multiple locations. Over all locations the self-pollinated plants (A129) averaged 78.3% oleic acid. The fatty acid
20 composition of the A129 for each Idaho trial location are summarized in Table X. In multiple location replicated yield trials, A129 was not significantly different in yield from the parent cultivar Westar.

The canola oil of A129, after commercial processing,
25 was found to have superior oxidative stability compared to Westar when measured by the Accelerated Oxygen Method (AOM), American Oil Chemists' Society Official Method Cd 12-57 for fat stability; Active Oxygen Method (revised 1989). The AOM of Westar was 18 AOM hours and for A129 was 30 AOM hours.

TABLE IX

Fatty Acid Composition of a High
Oleic Acid Canola Line Produced by Seed Mutagenesis

5	Genotype	Percent Fatty Acids					
		C _{16:0}	C _{18:0}	C _{18:1}	C _{18:2}	C _{18:3}	Sats
	Westar	3.9	1.9	67.5	17.6	7.4	7.0
	W7608.3 (M ₃)	3.9	2.4	71.2	12.7	6.1	7.6
10	W7608.3.5 (M ₄)	3.9	2.0	78.8	7.7	3.9	7.3
	A129.5.3 (M ₅)	3.8	2.3	75.6	9.5	4.9	7.6

15 Sats=Total Saturate Content

TABLE X

Fatty Acid Composition of a Mutant High
Oleic Acid Line at Different Field Locations in Idaho

20	Location	Percent Fatty Acids					
		C _{16:0}	C _{18:0}	C _{18:1}	C _{18:2}	C _{18:3}	Sats
	Burley	3.3	2.1	77.5	8.1	6.0	6.5
	Tetonia	3.5	3.4	77.8	6.5	4.7	8.5
	Lamont	3.4	1.9	77.8	7.4	6.5	6.3
25	Shelley	3.3	2.6	80.0	5.7	4.5	7.7

Sats=Total Saturate Content

The genetic relationship of the high oleic acid mutation A129 to other oleic desaturases was demonstrated in crosses made to commercial canola cultivars and a low linolenic acid mutation. A129 was crossed to the commercial

cultivar Global ($C_{16:0}$ - 4.5%, $C_{18:0}$ - 1.5%, $C_{18:1}$ - 62.9%, $C_{18:2}$ - 20.0%, $C_{18:3}$ - 7.3%). Approximately 200 F_2 individuals were analyzed for fatty acid composition. The results are summarized in Table XB. The segregation fit 1:2:1 ratio
5 suggesting a single co-dominant gene controlled the inheritance of the high oleic acid phenotype.

TABLE XB
Genetic Studies of A129 X Global

Genotype	$C_{18:0}$ Content (%)	Frequency	
		Observed	Expected
od-od-	77.3	43	47
od-od+	71.7	106	94
od+od+	66.1	49	47

15 A cross between A129 and IMC 01, a low linolenic acid variety ($C_{16:0}$ - 4.1%, $C_{18:0}$ - 1.9%, $C_{18:1}$ - 66.4%, $C_{18:2}$ - 18.1%, $C_{18:3}$ - 5.7%), was made to determine the inheritance of the oleic acid desaturase and linoleic acid desaturase.
20 In the F_1 hybrids both the oleic acid and linoleic acid desaturase genes approached the mid-parent values indicating a co-dominant gene actions. Fatty acid analysis of the F_2 individuals confirmed a 1:2:1:2:4:2:1:2:1 segregation of two independent, co-dominant genes (Table XC). A line was selected from the cross of A129 and IMC01 and designated as
25 IMC130 (ATCC deposit no. 75446) as described in U.S. Patent Application No. 08/425,108, incorporated herein by reference.

TABLE XC
Genetic Studies of A129 X IMC 01

		Frequency		
	Genotype	Ratio	Observed	Expected
5	od-od-ld-ld-	1	11	12
	od-od-ld-ld+	2	30	24
	od-od-ld+ld+	1	10	12
	od-od+ld-ld-	2	25	24
	od-od+ld-ld+	4	54	47
	od-od+ld+ld+	2	18	24
	od+od+ld-ld-	1	7	12
	od+od+ld-ld+	2	25	24
	od+od+ld+ld+	1	8	12

An additional high oleic acid line, designated
15 A128.3, was also produced by the disclosed method. A 50-seed bulk analysis of this line showed the following fatty acid composition: C_{16:0} - 3.5%, C_{18:0} - 1.8%, C_{18:1} - 77.3%, C_{18:2} - 9.0%, C_{18:3} - 5.6%, FDA Sats - 5.3%, Total Sats - 6.4%. This line also stably maintained its mutant fatty acid
20 composition to the M₇ generation. In multiple locations replicated yield trials, A128 was not significantly different in yield from the parent cultivar Westar.

A129 was crossed to A128.3 for allelism studies. Fatty acid composition of the F₂ seed showed the two lines
25 to be allelic. The mutational events in A129 and A128.3 although different in origin were in the same gene.

An additional high oleic acid line, designated M3028.-10 (ATCC 75026), was also produced by the disclosed method in Example 1. A 10-seed bulk analysis of this line showed the following fatty acid composition: C_{16:0} - 3.5%, C_{18:0} - 1.8%, C_{18:1} - 77.3%, C_{18:2} - 9.0%, C_{18:3} - 5.6%, FDA Saturates - 5.3%, Total Saturates - 6.4%. In a single location replicated yield trial M3028.10 was not

significantly different in yield from the parent cultivar Westar.

EXAMPLE 7

Low Linoleic Acid Canola

5 In the studies of Example 1, at the M₃ generation, 80 lines exceeded the lower statistical threshold for linoleic acid (\leq 13.2%). Line W12638.8 had 9.4% linoleic acid. At the M₄ and M₅ generations, its selfed progenies [W12638.8, since designated A133.1 (ATCC 40812)] continued
10 to exceed the statistical threshold for low C_{18:2} with linoleic acid levels of 10.2% and 8.4%, respectively. The fatty acid composition of this low linoleic acid mutant, which was stable to the M₇ generation under both field and greenhouse conditions, is summarized in Table XI. In
15 multiple location replicated yield trials, A133 was not significantly different in yield from the parent cultivar Westar. An additional low linoleic acid line, designated M3062.8 (ATCC 75025), was also produced by the disclosed method. A 10-seed bulk analysis of this line showed the
20 following fatty acid composition: C_{16:0} - 3.8%, C_{18:0} - 2.3%, C_{18:1} - 77.1%, C_{18:2} - 8.9%, C_{18:3} - 4.3%, FDA Sats-6.1%. This line has also stably maintained its mutant fatty acid composition in the field and greenhouse.

TABLE XI

Fatty Acid Composition of a Low
Linoleic Acid Canola Line Produced by Seed Mutagenesis

5	Genotype ^a	Percent Fatty Acids					
		C _{16:0}	C _{18:0}	C _{18:1}	C _{18:2}	C _{18:3}	Sats ^b
	Westar	3.9	1.9	67.5	17.6	7.4	7.0
	W12638.8 (M ₃)	3.9	2.3	75.0	9.4	6.1	7.5
10	W12638.8.1 (M ₄)	4.1	1.7	74.6	10.2	5.9	7.1
	A133.1.8 (M ₅)	3.8	2.0	77.7	8.4	5.0	7.0

15 ^aLetter and numbers up to second decimal point indicate the plant line. Number after second decimal point indicates an individual plant.

^bSats=Total Saturate Content

EXAMPLE 8

20 Low Linolenic and Linoleic Acid Canola

In the studies of Example 1, at the M₃ generation, 57 lines exceeded the lower statistical threshold for linolenic acid ($\leq 5.3\%$). Line W14749.8 had 5.3% linolenic acid and 15.0% linoleic acid. At the M₄ and M₅ generations, 25 its selfed progenies [W14749.8, since designated M3032 (ATCC 75021)] continued to exceed the statistical threshold for low C_{18:3} with linolenic acid levels of 2.7% and 2.3%, respectively, and for a low sum of linolenic and linoleic acids with totals of 11.8% and 12.5% respectively. The 30 fatty acid composition of this low linolenic acid plus linoleic acid mutant, which was stable to the M₅ generation under both field and greenhouse conditions, is summarized in

Table XII. In a single location replicated yield trial M3032 was not significantly different in yield from the parent cultivar (Westar).

TABLE XII

5 Fatty Acid Composition of a Low
Linolenic Acid Canola Line Produced by Seed Mutagenesis

Genotype	Percent Fatty Acids					
	C _{16:0}	C _{18:0}	C _{18:1}	C _{18:2}	C _{18:3}	Sats
10 Westar	3.9	1.9	67.5	17.6	7.4	7.0
W14749.8 (M ₃)	4.0	2.5	69.4	15.0	5.3	6.5
M3032.8 (M ₄)	3.9	2.4	77.9	9.1	2.7	6.4
15 M3032.1 (M ₅)	3.5	2.8	80.0	10.2	2.3	6.5

Sats=Total Saturate Content

EXAMPLE 9

20 The high oleic acid mutation of A129 was introduced into different genetic backgrounds by crossing and selecting for fatty acid and agronomic characteristics. A129 (now renamed IMC 129) was crossed to Legend, a commercial spring *Brassica napus* variety. Legend has the following fatty acid 25 composition: C_{16:0} - 3.8%, C_{18:0} - 2.1%, C_{18:1} - 63.1%, C_{18:2} - 17.8%, C_{18:3} - 9.3%. The cross and progeny resulting from were coded as 89B60303.

30 The F₁ seed resulting from the cross was planted in the greenhouse and self-pollinated to produce F₂ seed. The F₂ seed was planted in the field for evaluation. Individual plants were selected in the field for agronomic

characteristics. At maturity, the F_3 seed was harvested from each selected plant and analyzed for fatty acid composition.

Individuals which had fatty acid profiles similar to the high oleic acid parent (IMC 129) were advanced back to the field. Seeds (F_3) of selected individuals were planted in the field as selfing rows and in plots for preliminary yield and agronomic evaluations. At flowering the F_3 plants in the selfing rows were self-pollinated. At maturity the F_4 seed was harvested from individual plants to determine fatty acid composition. Yield of the individual selections was determined from the harvested plots.

Based on fatty acid composition of the individual plants and yield and agronomic characteristics of the plots F_4 lines were selected and advanced to the next generation in the greenhouse. Five plants from each selected line were self-pollinated. At maturity the F_5 seed was harvested from each and analyzed for fatty acid composition.

The F_5 line with the highest oleic fatty profile was advanced to the field as a selfing row. The remaining F_5 seed from the five plants was bulked together for planting the yield plots in the field. At flowering, the F_5 plants in each selfing-row were self-pollinated. At maturity the F_6 self-pollinated seed was harvest from the selfing row to determine fatty acid composition and select for the high oleic acid trait. Yield of the individual selections was determined from the harvested plots.

Fifteen F_6 lines having the high oleic fatty profile of IMC 129 and the desired agronomic characteristics were advanced to the greenhouse to increase seed for field trialing. At flowering the F_6 plants were self-pollinated. At maturity the F_7 seed was harvested and analyzed for fatty acid composition. Three F_7 seed lines which had fatty acid

profiles most similar to IMC 129 (Table XIII) were selected and planted in the field as selfing rows, the remaining seed was bulked together for yield trialing. The high oleic fatty acid profile of IMC 129 was maintained through seven 5 generations of selection for fatty acid and agronomic traits in an agronomic background of *Brassica napus* which was different from the parental lines. Thus, the genetic trait from IMC 129 for high oleic acid can be used in the development of new high oleic *Brassica napus* varieties.

10

TABLE XIII

Fatty Acid Composition of Advanced Breeding Generation
with High Oleic Acid Trait (IMC 129 X Legend)

F ₇ Selections of 89B60303	Fatty Acid Composition (%)				
	C _{16:0}	C _{18:0}	C _{18:1}	C _{18:2}	C _{18:3}
93.06194	3.8	1.6	78.3	7.7	4.4
93.06196	4.0	2.8	77.3	6.8	3.4
93.06198	3.7	2.2	78.0	7.4	4.2

20

The high oleic acid trait of IMC 129 was also introduced into a different genetic background by combining crossing and selection methods with the generation of dihaploid populations from the microspores of the F₁ hybrids. IMC 129 was crossed to Hyola 41, a commercial 25 spring *Brassica napus* variety. Hyola 41 has the following fatty acid composition: C_{16:0} - 3.8%, C_{18:0} - 2.7%, C_{18:1} - 64.9%, C_{18:2} - 16.2%, C_{18:3} - 9.1%. The cross and progeny resulting from the cross were labeled 90DU.146.

30

The F₁ seed was planted from the cross and a dihaploid (DH₁) population was made from the F₁ microspores using standard procedures for *Brassica napus*. Each DH₁ plant was self-pollinated at flowering to produce DH₁ seed.

At maturity the DH₁ seed was harvested and analyzed for fatty acid composition. DH₁ individuals which expressed the high oleic fatty acid profit of IMC 129 were advanced to the next generation in the greenhouse. For each individual 5 selected five DH₁ seeds were planted. At flowering the DH₂ plants were self-pollinated. At maturity the DH₂ seed was harvested and analyzed for fatty acid composition. The DH₂ seed which was similar in fatty acid composition to the IMC 129 parent was advanced to the field as a selfing row. The 10 remaining DH₂ seed of that group was bulked and planted in plots to determine yield and agronomic characteristics of the line. At flowering individual DH₃ plants in the selfing row were self-pollinated. At maturity the DH₃ seed was harvested from the individual plants to determine fatty acid 15 composition. Yield of the selections was determined from the harvested plots. Based on fatty acid composition, yield and agronomic characteristics selections were advanced to the next generation in the greenhouse. The DH₄ seed produced in the greenhouse by self-pollination was analyzed 20 for fatty acid composition. Individuals which were similar to the fatty acid composition of the IMC 129 parent were advanced to the field to test for fatty acid stability and yield evaluation. The harvested DH₅ seed from six locations maintained the fatty acid profile of the IMC 129 parent 25 (Table XIV).

TABLE XIV

Fatty Acid Composition of Advanced Dihaploid Breeding Generation with High Oleic Acid Trait (IMC 129 X Hyola 41)

5	DH5 of 90DU.146 at Multiple Locations	Fatty Acid Composition (%)				
		C _{16:0}	C _{18:0}	C _{18:1}	C _{18:2}	C _{18:3}
	Aberdeen	3.7	2.6	75.4	8.1	7.2
	Blackfoot	3.3	2.4	75.5	8.8	7.5
10	Idaho Falls	3.7	3.1	75.0	7.5	8.1
	Rexberg	3.9	3.7	75.3	7.0	6.5
	Swan Valley	3.5	3.4	74.5	7.0	7.3
	Lamont	3.9	2.8	72.0	10.1	8.4

EXAMPLE 10

15 Canola Line Q508

Seeds of the *B. napus* line IMC-129 were mutagenized with methyl N-nitrosoguanidine (MNNG). The MNNG treatment consisted of three parts: pre-soak, mutagen application, and wash. A 0.05M Sorenson's phosphate buffer was used to maintain pre-soak and mutagen treatment pH at 6.1. Two hundred seeds were treated at one time on filter paper (Whatman #3M) in a petri dish (100mm x 15mm). The seeds were pre-soaked in 15 mls of 0.05M Sorenson's buffer, pH 6.1, under continued agitation for two hours. At the end of the pre-soak period, the buffer was removed from the plate.

20 A 10mM concentration of MNNG in 0.05M Sorenson's buffer, pH 6.1, was prepared prior to use. Fifteen ml of 10m MNNG was added to the seeds in each plate. The seeds were incubated at 22°C±3°C in the dark under constant agitation for four (4) hours. At the end of the incubation period, the mutagen solution was removed.

25 The seeds were washed with three changes of distilled water at 10 minute intervals. The fourth wash was

for thirty minutes. This treatment regime produced an LD60 population.

Treated seeds were planted in standard greenhouse potting soil and placed into an environmentally controlled 5 greenhouse. The plants were grown under sixteen hours of light. At flowering, the racemes were bagged to produce selfed seed. At maturity, the M2 seed was harvested. Each M2 line was given an identifying number. The entire MNNG-treated seed population was designated as the Q series.

10 Harvested M2 seeds was planted in the greenhouse. The growth conditions were maintained as previously described. The racemes were bagged at flowering for selfing. At maturity, the selfed M3 seed was harvested and analyzed for fatty acid composition. For each M3 seed line, 15 approximately 10-15 seeds were analyzed in bulk as described in Example 1.

High oleic-low linoleic M3 lines were selected from the M3 population using a cutoff of >82% oleic acid and <50% linoleic. From the first 1600 M3 lines screened for fatty 20 acid composition, Q508 was identified. Table XV shows the fatty acid composition of Q508, Westar and IMC 129. The Q508 M3 generation was advanced to the M4 generation in the greenhouse. The M4 selfed seed maintained the selected high oleic-low linoleic acid phenotype (Table XVI).

25 Nine other M4 high-oleic low-linoleic lines were also identified: Q3603, Q3733, Q4249, Q6284, Q6601, Q6761, Q7415, Q4275, and Q6676. Some of these lines had good agronomic characteristics and an elevated oleic acid level in seeds of about 80% to about 84%.

TABLE XV
 Fatty Acid Composition of A129 and High
Oleic Acid M3 Mutant Q508

Line #	16:0	18:0	18:1	18:2	18:3
5 A129*	4.0	2.4	77.7	7.8	4.2
Q508	3.9	2.1	84.9	2.4	2.9

*Fatty acid composition of A129 is the average of 50 self-pollinated plants grown with the M3 population

10 M₄ generation Q508 plants had poor agronomic qualities in the field compared to Westar. Typical plants were slow growing relative to Westar, lacked early vegetative vigor, were short in stature, tended to be chlorotic and had short pods. The yield of Q508 was very low compared to Westar.

15 The M₄ generation Q508 plants in the greenhouse tended to be reduced in vigor compared to Westar. However, Q508 yields in the greenhouse were greater than Q508 yields in the field.

TABLE XVI
 Fatty Acid Composition of Seed Oil
from Greenhouse-Grown Q508, IMC129 and Westar.

Line	16:0	18:0	18:1	18:2	18:3	FDA Sats
IMC129 ^a	4.0	2.4	77.7	7.8	4.2	6.4
Westar ^b	3.9	1.9	67.5	17.6	7.4	>5.8
Q508 ^c	3.9	2.1	84.9	2.4	2.9	6.0

^aAverage of 50 self-pollinated plants

^bData from Example 1

^cAverage of 50 self-pollinated plants

M_4 generation Q508 plants were crossed to a dihaploid selection of Westar, with Westar serving as the female parent. The resulting F1 seed was termed the 92EF population. About 126 F1 individuals that appeared to have better agronomic characteristics than the Q508 parent were selected for selfing. A portion of the F₂ seed from such individuals was replanted in the field. Each F2 plant was selfed and a portion of the resulting F3 seed was analyzed for fatty acid composition. The content of oleic acid in F₃ seed ranged from 59 to 79%. No high oleic (>80%) individuals were recovered with good agronomic type.

TABLE XVII

LOCATION	SITE CHARACTERISTICS
BURLEY	Irrigated. Long season. High temperatures during flowering.
TETONIA	Dryland. Short season. Cool temperatures.
LAMONT	Dryland. Short season. Cool temperatures.
SHELLEY	Irrigated. Medium season. High temperatures during flowering.

Yield of the entries in the trial was determined by taking the statistical average of the four replications.

The Least Significant Difference Test was used to rank the entries in the randomized complete block design.

A portion of the F₂ seed of the 92EF population was planted in the greenhouse to analyze the genetics of the Q508 line. F₃ seed was analyzed from 380 F2 individuals.

The C_{18:1} levels of F₃ seed from the greenhouse experiment is

depicted in Figure 3. The data were tested against the hypothesis that Q508 contains two mutant genes that are semi-dominant and additive: the original IMC129 mutation as well as one additional mutation. The hypothesis also 5 assumes that homozygous Q508 has greater than 85% oleic acid and homozygous Westar has 62-67% oleic acid. The possible genotypes at each gene in a cross of Q508 by Westar may be designated as:

10 AA = Westar Fad2^a
 BB = Westar Fad2^b
 aa = Q508 Fad2^{a-}
 bb = Q508 Fad2^{b-}

Assuming independent segregation, a 1:4:6:4:1 ratio of phenotypes is expected. The phenotypes of heterozygous 15 plants are assumed to be indistinguishable and, thus, the data were tested for fit to a 1:14:1 ratio of homozygous Westar: heterozygous plants: homozygous Q508.

	Phenotypic Ratio	# of Westar Alleles	Genotype
20	1	4	AABB (Westar)
	4	3	AABb, AaBB, AABb, AaBB
	6	2	AaBb, AAAb, AaBb, AaBb, aaBB, AaBb
	4	1	Aabb, aaBb, Aabb, aaBb
	1	0	aabb (Q508)

25 Using Chi-square analysis, the oleic acid data fit a 1:14:1 ratio. It was concluded that Q508 differs from Westar by two major genes that are semi-dominant and additive and that segregate independently. By comparison, the genotype of IMC129 is aaBB.

30 The fatty acid composition of representative F3 individuals having greater than 85% oleic acid in seed oil

13
are shown in Table XVIII. The levels of saturated fatty acids are seen to be decreased in such plants, compared to Westar.

TABLE XVIII

5 92EF F₃ Individuals with >85% C_{18:1} in Seed Oil

F3 Plant Identifier	Fatty Acid Composition (%)						
	C16:0	C18:0	C18:1	C18:2	C18:3	FDASA	
10	+38068	3.401	1.582	85.452	2.134	3.615	4.983
	+38156	3.388	1.379	85.434	2.143	3.701	4.767
	+38171	3.588	1.511	85.289	2.367	3.425	5.099
	+38181	3.75	1.16	85.312	2.968	3.819	4.977
	+38182	3.529	0.985	85.905	2.614	3.926	4.56
	+38191	3.364	1.039	85.737	2.869	4.039	4.459
	+38196	3.557	1.182	85.054	2.962	4.252	4.739
	+38202	3.554	1.105	86.091	2.651	3.721	4.713
	+38220	3.093	1.16	86.421	1.931	3.514	4.314
	+38236	3.308	1.349	85.425	2.37	3.605	4.718
20	+38408	3.617	1.607	85.34	2.33	3.562	5.224
	+38427	3.494	1.454	85.924	2.206	3.289	4.948
	+38533	3.64	1.319	85.962	2.715	3.516	4.959

EXAMPLE 11

Leaf and Root Fatty Acid Profiles of Canola

Lines IMC-129, Q508, and Westar

Plants of Q508, IMC 129 and Westar were grown in the greenhouse. Mature leaves, primary expanding leaves, petioles and roots were harvested at the 6-8 leaf stage, 5 frozen in liquid nitrogen and stored at -70°C. Leaf ^{Lipid} lipid extracts were analyzed by GLC as described in Example 1. The fatty acid profile data are shown in Table XIX.

The data in Table XIX indicate that total leaf lipids in Q508 are higher in C_{18:1} content than the C_{18:2} plus 10 C_{18:3} content. The reverse is true for Westar and IMC 129. The difference in total leaf lipids between Q508 and IMC129 is consistent with the hypothesis that a second Fad2 gene is mutated in Q508.

The C_{16:3} content in the total lipid fraction was 15 about the same for all three lines, suggesting that the plastid FadC gene product was not affected by the Q508 mutations. To confirm that the FadC gene was not mutated, chloroplast lipids were separated and analyzed. No changes in chloroplast C_{16:1}, C_{16:2} or C_{16:3} fatty acids were detected 20 in the three lines. The similarity in plastid leaf lipids among Q508, Westar and IMC129 is consistent with the hypothesis that the second mutation in Q508 affects a microsomal Fad2 gene and not a plastid FadC gene.

TABLE XIX

	MATURE LEAF			EXPANDING LEAF			PETIOLE			ROOT			
	West.	129	3Q508	West.	129	3Q508	West.	129	3Q508	West.	129	3Q508	
5	16:0	12.1	11.9	10.1	16.4	16.1	11.3	21.7	23.5	11.9	21.1	21.9	12.0
	16:1	0.8	0.6	1.1	0.7	0.6	1.1	1.0	1.3	1.4	-	-	-
	16:2	2.3	2.2	2.0	2.8	3.1	2.8	1.8	2.2	1.8	-	-	-
	16:3	14.7	15.0	14.0	6.3	5.4	6.9	5.7	4.6	5.7	-	-	-
	18:0	2.2	1.6	1.2	2.5	2.8	1.5	3.7	4.0	1.6	3.6	2.9	2.5
	18:1	2.8	4.9	16.7	3.8	8.3	38.0	4.9	12.9	46.9	3.5	6.1	68.8
	18:2	12.6	11.5	6.8	13.3	13.8	4.9	20.7	18.3	5.2	28.0	30.4	4.4
	18:3	50.6	50.3	46.0	54.2	50.0	33.5	40.4	33.2	25.3	43.8	38.7	12.3

10

EXAMPLE 12

Sequences of Mutant and Wild-Type Delta-12 Fatty Acid Desaturases from *B. napus*

Primers specific for the FAD2 structural gene were used to clone the entire open reading frame (ORF) of the D and F 12-DES genes by reverse transcriptase polymerase chain reaction (RT-PCR). RNA from seeds of IMC129, Q508 and Westar plants was isolated by standard methods and was used as template. The RT-amplified fragments were used for nucleotide sequence determination. The DNA sequence of each gene from each line was determined from both strands by standard dideoxy sequencing methods.

Sequence analysis revealed a G to A transversion at nucleotide 316 (from the translation initiation codon) of the D gene in both IMC129 and Q508, compared to the sequence of Westar. The transversion changes the codon at this position from GAG to AAG and results in a non-conservative substitution of glutamic acid, an acidic residue, for lysine a basic residue. The presence of the same mutation in both

lines was expected since the Q508 line was derived from IMC129. The same base change was also detected in Q508 and IMC129 when RNA from leaf tissue was used as template.

The G to A mutation at nucleotide 316 was confirmed 5 by sequencing several independent clones containing fragments amplified directly from genomic DNA of *IMC129* and Westar. These results eliminated the possibility of a rare mutation introduced during reverse transcription and PCR in the RT-PCR protocol. It was concluded that the *IMC129* 10 mutant is due to a single base transversion at nucleotide 316 in the coding region of the D gene of rapeseed microsomal delta 12-desaturase.

A single base transition from T to A at nucleotide 515 of the F gene was detected in Q508 compared to the 15 Westar sequence. The mutation changes the codon at this position from CTC to CAC, resulting in the non-conservative substitution of a non-polar residue, leucine, for a polar residue, histidine, in the resulting gene product. No mutations were found in the F gene sequence of *IMC129* 20 compared to the F gene sequence of Westar.

These data support the conclusion that a mutation in a delta-12 desaturase gene sequence results in alterations in the fatty acid profile of plants containing such a mutated gene. Moreover, the data show that when a plant 25 line or species contains two delta-12 desaturase loci, the fatty acid profile of an individual having two mutated loci differs from the fatty acid profile of an individual having one mutated locus.

The mutation in the D gene of *IMC129* and Q508 mapped 30 to a region having a conserved amino acid motif (His-Xaa-Xaa-Xaa-His) found in cloned delta-12 and delta-15 membrane bound-desaturases (Table XX).

Table XX

Alignment of Amino Acid Sequences
of Cloned Canola Membrane Bound-Desaturases

	Desaturase Gene	Sequence ^a	Position ^b
5	Canola-fad2-D(129)	A H KCGH	109
	Canola-FAd2-D	AHECGH	109
	Canola-FAd2-F	AHECGH	109
	Canola Fadc	<u>G</u> HDCAH	170
	Canola-Fad3	<u>G</u> HDCGH	96
10	Canola-FadD	<u>G</u> HDCGH	125

(FadD = Plastid delta 15, Fad3 = Microsomal delta-15),
(FadC = Plastid delta-12, Fad2 = Microsomal delta-12)

^a One letter amino acid code; conservative substitutions are underlined; non-conservative substitutions are in bold.

^b position of first amino acid in gene product.

EXAMPLE 13

Transcription and Translation of Microsomal Delta-12
Fatty Acid Desaturases

Transcription *in vivo* was analyzed by RT-PCR analysis of stage II and stage III developing seeds and leaf tissue. The primers used to specifically amplify 12-DES F gene RNA from the indicated tissues were sense primer 5'-GGATATGATGATGGTCAAAGA-3' and antisense primer 5'-TCTTCACCATCATCATATCC-3'. The primers used to specifically amplify 12-DES D gene RNA from the indicated tissues were sense primer 5'-GTTATGAAGCAAAGAAC-3' and antisense primer 5'-GTTTCTTCTTGCTTCATAAC-3'. The results indicated that mRNA of both the D and F gene was expressed in seed and leaf tissues of IMC129, Q508 and wild type Westar plants.

In vitro transcription and translation analysis showed that a peptide of about 46 kD was made. This is the expected size of both the D gene product and the F gene product, based on sum of the deduced amino acid sequence of each gene and the cotranslational addition of a microsomal membrane peptide.

These results rule out the possibility that non-sense or frameshift mutations, resulting in a truncated polypeptide gene product, are present in either the mutant D gene or the mutant F gene. The data, in conjunction with the data of Example 12, support the conclusion that the mutations in Q508 and IMC129 are in delta-12 fatty acid desaturase structural genes encoding desaturase enzymes, rather than in regulatory genes.

15

EXAMPLE 14

Development of Gene-Specific PCR Markers

Based on the single base change in the mutant D gene of IMC129 described in above, two 5' PCR primers were designed. The nucleotide sequence of the primers differed only in the base (G for Westar and A for IMC129) at the 3' end. The primers allow one to distinguish between mutant *Can-Fad2-D-129* and wild-type *Can-Fad2-D* alleles in a DNA-based PCR assay. Since there is only a single base difference in the 5' PCR primers, the PCR assay is very sensitive to the PCR conditions such as annealing temperature, cycle number, amount, and purity of DNA templates used. Assay conditions have been established that distinguish between the mutant gene and the wild type gene using genomic DNA from IMC129 and wild type plants as templates. Conditions may be further optimized by varying PCR parameters, particularly with variable crude DNA samples. A PCR assay distinguishing the single base

mutation in IMC129 from the wild type gene along with fatty acid composition analysis provides a means to simplify segregation and selection analysis of genetic crosses involving plants having a delta-12 fatty acid desaturase

5 mutation.

To the extent not already indicated, it will be understood by those of ordinary skill in the art that any one of the various specific embodiments herein described and illustrated may be further modified to incorporate features

10 shown in other of the specific embodiments.

The foregoing detailed description has been provided for a better understanding of the invention only and no unnecessary limitation should be understood therefrom as some modifications will be apparent to those skilled in the

15 art without deviating from the spirit and scope of the appended claims.